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Genetic Variation and Phylogeographical Patterns in *Alsophila podophylla* from Southern China Based on cpDNA *atp*B-*rbc*L Sequence Data

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ABSTRACT.—Chloroplast DNA (cpDNA) *atp*B-*rbc*L intergenic spacers of individuals of *Alsophila podophylla*, collected from eight relict populations distributed in Hainan and Guangdong Province, southern China, were sequenced. Sequence sizes were 726 or 727bp. Base composition had a high A+T content of 62.67–63.00%. Sequences were assessed as evolutionarily neutral (Tajima's criterion D = –0.80683, P > 0.10 and Fu and Li's test D* = 1.42648, P > 0.05; F* = 0.76638, P > 0.10). Eight haplotypes were identified based on a statistical parsimony algorithm. A high level of haplotype diversity ($h = 0.618$) and a low nucleotide diversity ($Dij = 0.00208$) were detected in *A. podophylla*. Populations from Hainan shared common haplotypes with those from Guangdong. A network and a NJ tree constructed from cpDNA haplotypes both suggested a close genetic relationship among populations distributed in Hainan and Guangdong. Observed F_{ST} (=0.10537), gene flow *Nm* (=2.12), AMOVA (Only 0.49% of variation was partitioned among regions, P = 0.09), and DNA divergence data consistently indicated that no geographical differentiation occured at the interregional level. Geographic isolation has not yet resulted in population differentiations within *A. podophylla* populations in Hainan and Guangdong. Phylogeographical patterns of *atp*B-*rbc*L haplotypes demonstrate a 'star-like' feature, which means that populations of *A. podophylla* have experienced population expansion, and, since then there has been insufficient time to form a more complicated population structure. The majority of haplotypes coalesced near the tip of the NJ tree, indicating recent coalescence events as well. Moreover, a demographic signature of population expansion was detected by mismatch distribution analysis of *atp*B-*rbc*L sequences of *A. podophylla*.

Alsophila podophylla Hook. is a small tree fern belonging to *Alsophila* subgenus *Gymnosphaera* of the Cyatheaceae (Xia, 1989). With erect or tilted trunks, it averages 2 m in height and has 2-pinnate-pinnatifid fronds at the apex; its sori are round, lack indusia, and are located on raised receptacles positioned at the base of veins (Chen, 1964). Cyatheoids were globally distributed during the middle Mesozoic. Due to subsequent geologic and climatic changes, many of their ancestral species became extinct. Only some survived in tropical and subtropical montane zones with relictual distributions (Lucansky, 1974; Willis and McElwain, 2002). Extant *A. podophylla* are mainly restricted to rain forests at altitudes of 350–700 m, growing on shaded meadows, wetlands or by streamsides. In China, natural populations of *A. podophylla* are recorded from Guizhou, Yunnan, Hainan, and Guangdong Provinces (Xia, 1989; Chen, 1964),

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forming valuable materials for research on population genetic structure and phylogeography of pteridophytes.

In seed plants, factors determining genetic structure of populations include mating system, gene flow, selection pressure, mutation, genetic drift, intraspecific phylogeny, evolutionary history, life history, and physical features of the habitat (Loveless and Hamrick, 1984). Whether these are also determinants of population structure in pteridophytes remains unclear. Recently gene genealogies and coalescence theory have produced powerful methodologies to investigate population genetic variation and differentiation (Castelloe and Templeton, 1994). Their combined applications have proved invaluable for uncovering information about population formation, distribution, and expansion (Pages and Holmes, 1998). Chloroplast DNA (cpDNA) noncoding spacers have been frequently utilized to survey population genetic variation and phylogeography of plants (Lu et al., 2002; Huang et al., 2001). Their uniparental inheritance, nearly neutral, fast evolution is well suited for reconstructing intraspecific phylogeographical patterns (Ferris et al., 1998). In addition, DNA sequencing can avoid length homoplasies which usually occur when using restriction fragment length polymorphism (RFLP) and PCR-based fingerprinting methods, and improves the level of resolution when sequence data are used to estimate population genetic structure and gene flow (Chiang et al., 2001).

In this study, sequence variation of haplotypes of cpDNA *atp*B-*rbc*L intergenic spacers were employed to examine the population genetic structure and phylogeographical pattern of eight relict *A. podophylla* populations distributed across Hainan and Guangdong Provinces in southern China. The goals of this investigation were: (i) to assess levels of genetic diversity and their hierarchical apportionment; (ii) to determine whether geographic differentiation has occurred among these populations at the inter-region level; and (iii) to tentatively identify the factors influencing population genetic structure.

Materials and Methods

Samples of *A. podophylla* were collected from eight wild populations distributed in Guangdong and Hainan Province throughout southern China (Table 1). Populations FKHDC, FKHDT, FKHXU, and FKHXD grew by streamsides or in ravines, ZQDHG and ZQDHJ from Dinghushan Arboretum; HNWZS by a river at 850 m, and HNDLS by a waterfall at 700 m in a montane forest. Young and healthy leaves were sampled from randomly selected individuals with intervals of at least 5 m and immediately preserved in silica gel. All samples were stored at –20°C until being processed. Voucher specimens have been deposited at the herbarium of Sun Yat-sen University (SYS), Guangzhou, China.

Total genomic DNA was extracted from ground tissue following modified CTAB protocols (Su et al., 1998). DNA concentration and purity were determined by measuring UV absorption using a Pharmacia 2000 UV/Visible spectrophotometer. DNA intactness was assessed by 0.8% agarose gel

TABLE 1. Sampled populations of *A. podophylla* and GenBank accession numbers of haplotypes.

Populations	Localities	Sample size	Accession number
Guangdong Province			
FKHDC	Dachong, Heishiding, Fengkai	9	AY304353
FKHDT	Dutian, Heishiding, Fengkai	10	AY304354
FKHXU	Xuesishangyou, Heishiding, Fengkai	12	AY304355-58
FKHXD	Xuesixiayou, Heishiding, Fengkai	10	AY304359
ZQDHG	Guikeng, Dinghushan, Zhaoqing	12	AY304344-47
ZQDHJ	Jifenglin, Dinghushan, Zhaoqing	15	AY304348-52
Hainan Province			
HNWZS	Wuzhishan, Wuzhishan natural reserve	15	AY304338-42
HNDLS	Diaoluoshan, Baishuiling natural reserve	8	AY304343

electrophoresis. PCR® was performed in a reaction volume of 100 μl using 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM of dNTP, 50 ng template DNA, 2U *Taq* polymerase, and 40 pmol of each primer. The primers of Chiang et al. (1998) were used to amplify the *apt*B-*rbc*L noncoding spacer of cpDNA: Primer1–5′-ACATCKARTACKGGACCAATAA-3′, Primer2–5′-AACACCAGCTTTRAATCCAA-3′. Primers were synthesized by Shanghai Bioasia Biotech Ltd., China. The thermocycling profile consisted of 3 min at 94°C, 30 cycles of 40 s at 94°C, 50 s at 50°C, 80 s at 72°C, and an additional extension for 7 min at 72°C. The size of the PCR products was determined by agarose electrophoresis. DNA sequences were determined by either cycle sequencing or by sequencing cloned PCR products. For DNAs that could be directly sequenced, 5μl PCR product was applied to cycle sequencing by using the same primers as in the PCR reaction. PCR products were purified by electrophoresis on a low melting 1.0% agarose gel. The desired DNA band was cut and recovered using UNIQ-10 kit (Shanghai Bioengineering Ltd., China). Purified PCR product was ligated to a pMD18-T vector and then was used to transform competent *E coli* cells DH-5α. Positive clones were identified by PCR. Purified plasmid DNA was sequenced in both directions by standard methods on an ABI 377 automated sequencer. Primers M13F and M13R located on pMD18-T vector were utilized for sequence determination.

The *atp*B-*rbc*L intergenic spacer sequences of haplotypes and outgroups were registered to Genbank with accession numbers of AY304338–AY304359 and AY796292–AY796295, respectively. Sequences were aligned with the program CLUSTAL X (Thompson et al., 1997). Length variation and nucleotide composition were calculated using BioEdit (Hall, 1999). Tests of neutrality were performed using Tajima (1989)'s D as well as Fu and Li (1993)' D* test. Haplotype diversity (*h*) and nucleotide diversity (*Dij*) were estimated using DnaSP program (Rozas and Rozas, 1999).

Relationships of haplotypes have often been reconstructed in phylogeographical studies. However, as evolutionary relationships above and below the

species level are fundamentally different, it is controversial whether algorithms developed to analyze interspecific phylogeny are suitable for intraspecific assay. Thus, networks rather than trees have been suggested to be the represent relationships of haplotypes (Widmer and Baltisberger, 1999). Templeton et al. (1992) established a statistical parsimony algorithm to reconstruct genealogy. This method first defines the uncorrected distance above which the parsimony criterion is violated with more than a 5% probability (parsimony limit), then all haplotyes are linked starting with the smallest distances and ending either when all haplotyes are connected or the distance corresponding to the parsimony limit has been reached. We performed the statistical parsimony algorithm with the aid of TCS: a computer program to estimate gene genealogies (Templeton et al., 1992). For neighbour-joining analysis, PHYLIP (Felsenstein, 1995) was employed by calculating Kimura 2-parameter distance. Confidence of the reconstructed clades was tested by bootstrapping with 1000 replicates. Investigation on molecular phylogeny of Cyatheaceae indicates that clade consisting of *Cyathea pectinata* Ching et S. H. Wu, *Cyathea pseudogigantea* Ching et S. H. Wu, *Cyathea gigantea* (Wall.) Holtt., and *Cyathea tinganensis* Ching et S. H. Wu forms the sister group of *Alsophila podophylla* Hook. (Wang et al., 2003). Therefore, the former four species were utilized as outgroups when performing analysis.

Gene flow within and among populations was approximated as *Nm*, the number of female migrants per generation between populations. *Nm* was estimated using the expression $F_{ST} = 1/(1+2Nm)$ where N is the female effective population size and m is the female migration rate (Slatkin, 1993). ARLEQUIN (Schneider et al., 2000) was used to deduce the molecular variance partition within and among populations (regions) based on square Euclidean distances. A Mantel test was implemented to determine whether pairwise values of F_{ST} were related to geographic distances between populations (Mantel, 1967). Historic demographic expansions were detected by examination of mismatch distributions (Rogers and Harpending, 1992). Concordance of our data with the distribution underlying the sudden-expansion model was assessed by means of a least-squares approach (Rogers, 1995; Schneider and Excoffier, 1999).

Results

In this study, cpDNA *atp*B-*rbc*L intergenic spacers of eight relict populations of *A. podophylla* were determined by cycle sequencing or sequencing cloned PCP products. Sequence sizes were 726 bp except for FKHXU04 whose length was 727 bp due to a single T insertion at site 266. A and T are common in the chloroplast sequence, with A-T ratios of 62.67–63.00%; this is consistent with the nucleotide composition of most noncoding regions and pseudogenes. Sequence variation demonstrated non-significant deviation to expectations of neutrality, both by Tajima's criterion ($D = -0.80683$, $P > 0.10$) and Fu and Li's test ($D^* = 1.42648$, $P > 0.05$; $F^* = 0.76638$, $P > 0.10$). Eight haplotypes of cpDNA *atp*B-*rbc*L spacers were identified in *A. podophylla* by running TCS.

Haplotypes, HNWZS01-05, HNDLS01, ZQDHG01-04, ZQDHJ01-03, ZQDHJ05, and FKHXU03, are common to the Hainan and Guangdong regions (Fig. 1).

A high level of haplotype diversity ($h = 0.618$) and a low nucleotide diversity ($Dij = 0.00208$) were detected within *A. podophylla*. At the population level, haplotype diversity and nucleotide diversity of ZQDHJ and FKHXU are 0.343, 0.00094 and 0.682, 0.00113, respectively; the rest of the populations were homogeneous. At the regional level, haplotype diversity and nucleotide diversity of populations in Guangdong are 0.742 and 0.00273; those from Hainan were homogeneous.

Figure 1 depicts the network established by statistical parsimony algorithm based on haplotypes of cpDNA *atp*B-*rbc*L intergenic spacers of *A. podophylla* and outgroups. The network shows a branched 'star-like' pattern (Pages and Holmes, 1998). Haplotyes of FKHXU01, FKHXU02, FKHXU04, and FKHDC01 coalesced to the central haplotye by one mutational step, whereas FKHDT01 and ZQDHJ04 by two mutational steps, and FKHXD01 by four mutational steps. No geographic differentiation was uncovered between Hainan and Guangdong populations of *A. podophylla*.

The neighbour-joining (NJ) analysis of haplotypes was also conducted. Figure 2 shows that haplotypes of *A. podophylla* formed a monophyletic group within which FKHXD01 diverges first followed by FKHXU01 and FKHXU04. Most of the haplotypes coalesced near the tip of the tree. Sequences from the same populations were never grouped into a monophyletic clade (Fig. 2). Branches from different populations were intermixed, indicating a certain amount of gene flow between them.

Population structure of *A. podophylla* was assessed based on sequence variation in the cpDNA *atp*B-*rbc*L noncoding spacer. Non-significant differentiation between regions of Hainan and Guangdong was revealed by the estimates of F_{ST} (=0.10537) and *Nm* (=2.12). Hierarchical analyses of sequence differences under AMOVA indicated that 14.74% of the molecular variance can be attributed to differences within populations ($P < 0.001$), 84.76% attributed among populations within regions ($P < 0.001$); whereas only 0.49% of molecular variance attributed to difference among regions ($P = 0.09$, Table 2). No differences were detected between populations in Hainan. The average number of nucleotide differences and the average number of nucleotide substitution between populations in Guangdong range from 0.400 to 6.000 and 0.00055 to 0.00826, respectively (Table 3). At the interregional level, the corresponding values between Hainan and Guangdong are 1.106 and 0.00152, evidently not surpassing the above divergence range estimated from populations restricted in Guangdong.

Pairwise estimates of F_{ST} between populations showed a nonsignificant relationship with geographic distance ($r = 0.18$, $P = 0.683$); thus, the "isolation by distance" model (Wright, 1943) was not supported here. The mismatch distribution of cpDNA *atp*B-*rbc*L noncoding spacer sequences of *A. podophylla* closely matched to expectations under the sudden-expansion model (Fig. 3). The raggedness index (Rogers and Harpending, 1992) was low and not significantly different from expectation ($R = 0.049$, $P = 0.910$).

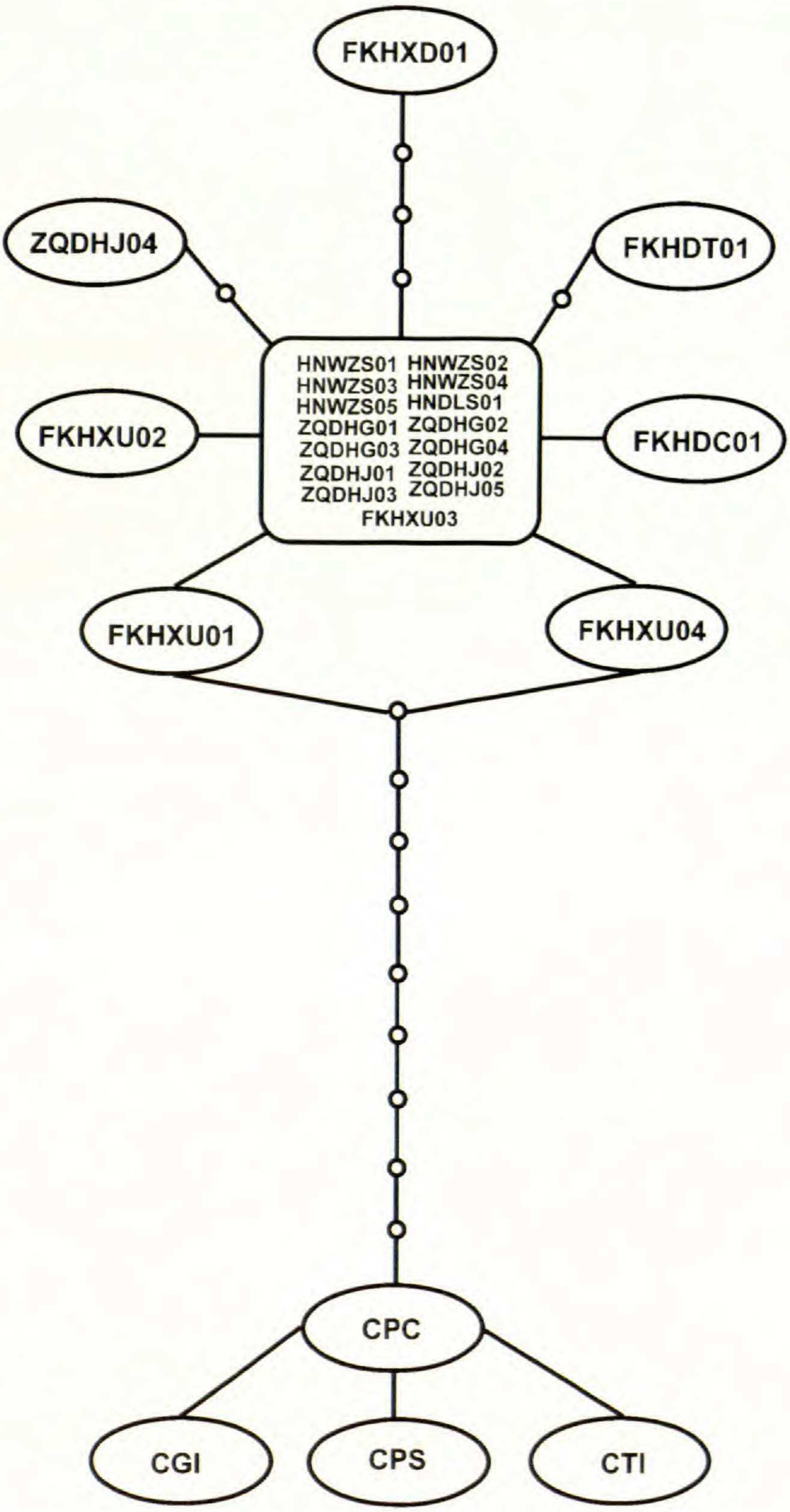

FIG. 1. Network relating haplotypes of cpDNA *atp*B-*rbc*L intergenic spacers in populations of *A. podophylla* by statistical parsimony algorithm. *Cyathea pectinata* (CPC), *Cyathea pseudogigantea* (CPS), *Cyathea gigantea* (CGI), and *Cyathea tinganensis* (CTI) were used to root the tree. Missing intermediates are indicated by circles. Each branch between two (sampled or missing) haplotypes indicates a single mutational step.

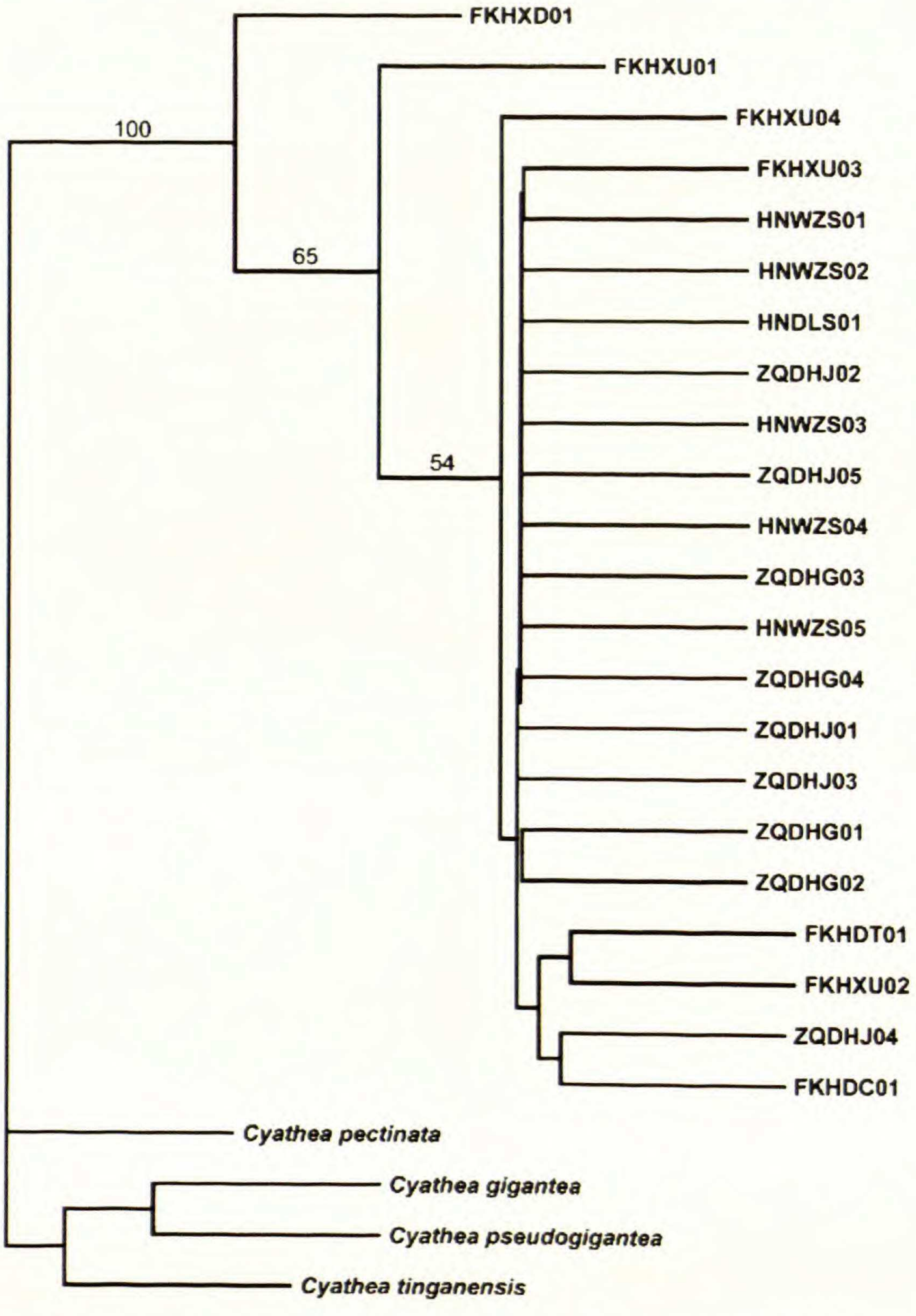

FIG. 2. Neighbour-joining tree of haplotypes of cpDNA *atp*B-*rbc*L intergenic spacers of *A. podophylla*, rooted using *Cyathea pectinata*, *Cyathea gigantea*, *Cyathea pseudogigantea* and *Cyathea tinganensis* as outgroups. Numbers above branches indicate the bootstrap values of 1000 replicates.

TABLE 2. Analysis of molecular variance (AMOVA) for populations of *A. podophylla* based on cpDNA *atp*B-*rbc*L intergenic spacers.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-Value
Among regions	1	9.650	0.00466	0.49	=0.09
Among populations within regions	6	54.822	0.80011	84.76	<0.001
Within populations	83	11.550	0.13916	14.74	<0.001
Total	90	76.022	0.94393		

DISCUSSION

Effects of natural selection on the evolution of *atp*B-*rbc*L intergenic sequences of *A. podophylla* were tested by both Tajima's (1989) and Fu and Li's (1993) methods. Results showed that the observed extent of sequence divergence was in agreement with the predictions under the neutral theory. Thus, in terms of evolution, the spacer is neutral. This fact makes *atp*B-*rbc*L spacer suitable for inferring population demographic histories based on gene genealogies because the neutral mutations do not alter the fitness of the individual, the number of offspring, or lineages (Nei and Kumar, 2000).

Although DNA sequencing has allowed for more of the genetic diversity within populations to be resolved, simple 'summary statistics', such as F_{ST} ignore much of the information in the data. Thus it is hard to distinguish among similar patterns of variation generated by very different evolutionary processes (Pages and Holmes, 1998). Gene genealogies and the coalescent analysis are increasingly utilized in population genetic analysis. In this research, both a network and a NJ tree were constructed from cpDNA *atp*B-*rbc*L haplotypes. Populations from Hainan (both HNWZS and HNDLS) shared common haplotypes with Guangdong populations (ZQDHG, ZQDHJ, and FKHXU; Fig. 1). Haplotypes from Hainan and Guangdong populations were dispersed among the branches in the NJ tree, neither forming as a monophyletic group (Fig. 2). These results suggest a close genetic relationship among the populations. Additionally, observed F_{ST} value (=0.10537), gene flow *Nm*

TABLE 3. Average number of nucleotide differences (below diagonal) and average number of nucleotide substitutions per site (above diagonal) between 8 populations of *A. podophylla*.

Populations	ZQDHG	ZQDHJ	FKHDC	FKHXU	FKHXD	FKHDT	HNWZS	HNDLS
ZQDHG		0.00055	0.00138	0.00069	0.00551	0.00275	0.00000	0.00000
ZQDHJ	0.400		0.00193	0.00124	0.00606	0.00331	0.00055	0.00055
FKHDC	1.000	1.400		0.00207	0.00689	0.00413	0.00138	0.00138
FKHXU	0.500	0.900	1.500		0.00620	0.00344	0.00069	0.00069
FKHXD	4.000	4.400	5.000	4.500		0.00826	0.00551	0.00551
FKHDT	2.000	2.400	3.000	2.500	6.000		0.00275	0.00275
HNWZS	0.000	0.400	1.000	0.500	4.000	2.000		0.00000
HNDLS	0.000	0.400	1.000	0.500	4.000	2.000	0.000	

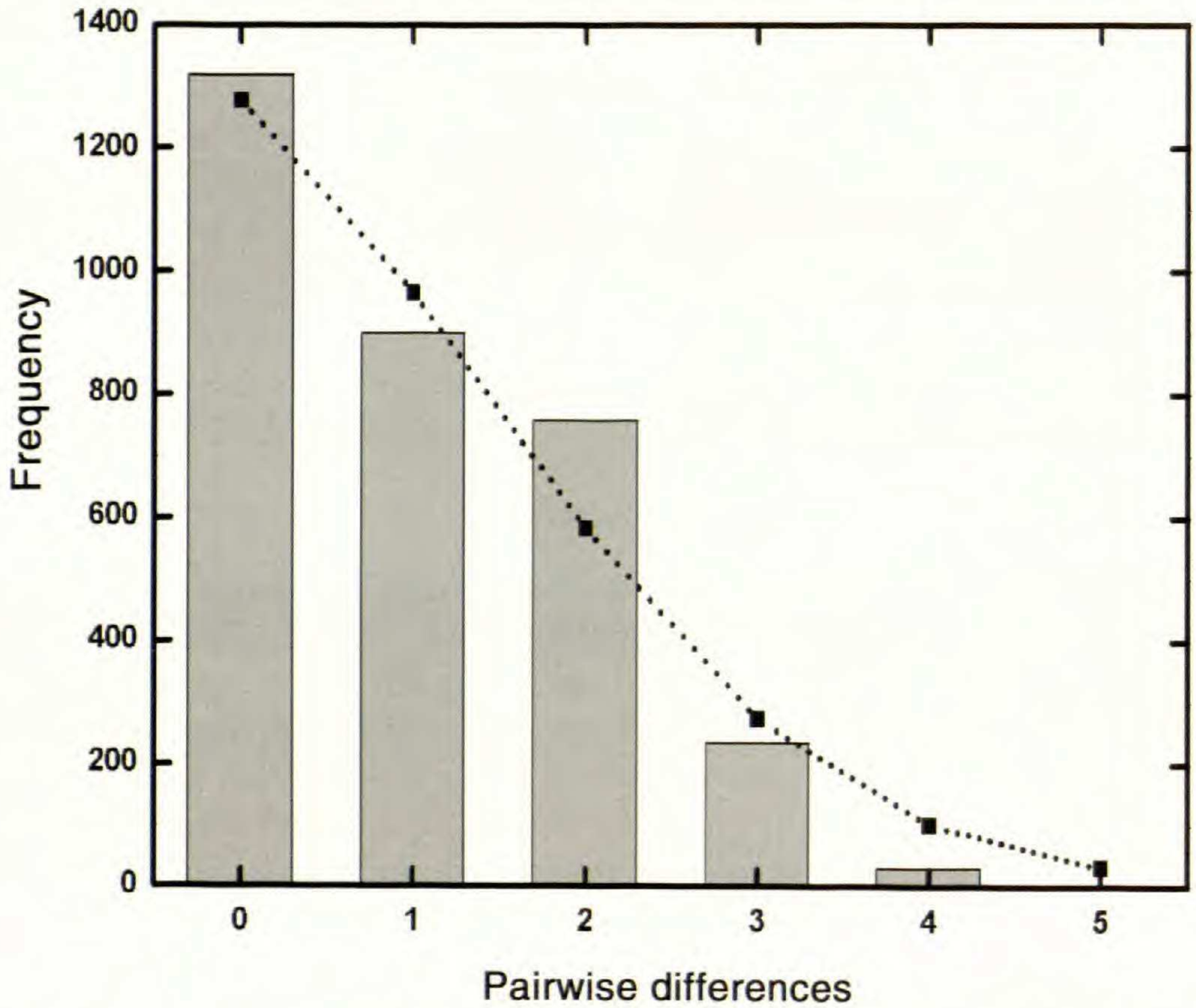

FIG. 3. Mismatch distribution across populations of *A. podophylla* based on cpDNA *atp*B-*rbc*L intergenic spacers. The abscissa represents number of pairwise differences and the ordinate represents the frequency of observations. The vertical bars are the observed distribution of mismatches and the dotted line represents the expected distribution under the sudden-expansion model of Rogers (1995) as modified by Schneider and Excoffier (1999).

(=2.12), AMOVA (0.49% of variation among regions, P = 0.09; Table 2), and DNA divergence data consistently indicated that no geographic differentiation occurred between Hainan and Guangdong populations. Geologically, Hainan was separated from the Chinese mainland during the late Tertiary and the early Quaternary by shifts in the location of regional land masses due to plate tectonics and subsequent division by rising sea levels (Xing et al., 1995). In the late Pleistocene, Hainan was again linked to the mainland possibly due to global sea dropping; but, with the advent of following warm period in the Holocene, Hainan again became isolated (Xing et al., 1995). Since then Hainan and Guangdong were separated by the Qiongzhou strait, with a width of 20–40 km. Interestingly, this research demonstrates that vicariant events have not yet generated interregional population differentiation in *A. podophylla*.

High gene flow, *Nm* of 2.12, was detected among populations of Hainan and Guangdong. But, considering the fragmentation of modern habitats, the constraint of migratory capabilities of spores and the fragility of spore vitality of cyatheoids (e.g. loss of vitality around 8 days; Cheng et al., 1990), we would not propose efficient ongoing gene flow between regions. Instead, we suggest that high *Nm* values are likely to represent historical migration events (Lu et al.,

2001). In contrast to other studies on population variability based on *atp*B-*rbc*L intergenic spacer data (e.g. *Cycas taitungensis*, Huang et al., 2001; *Michelia formosana*, Lu et al., 2002; *Dunnia sinensis*, Ge et al., 2002; *Trigonobalanus verticillata*, Kamiya et al., 2002; and *Aucuba japonica*, Ohi et al., 2003), a high level of haplotype diversity ($h = 0.618$) and a low nucleotide diversity ($Dij = 0.00208$) were revealed in *A. podophylla*. This suggests rapid demographic expansion from a small effective population size (Avise, 2000). Examination of frequency distributions of pairwise differences of *atp*B-*rbc*L sequences (Fig. 3) also suggests a recent demographic expansion across *A. podophylla* populations (Hundertmark, 2002). Furthermore, the phylogenetic pattern of *atp*B-*rbc*L haplotypes demonstrated a 'star-like' distribution, with short branch lengths, around a central core of haplotypes (Fig. 1). This relatively simple pattern suggests that populations of *A. podophylla* preserved in 'refugia' have experienced population expansion after glaciations, and since then there has been insufficient time to form a more complicated population structure (Pages and Holmes, 1998). In the NJ tree, a majority of the haplotypes coalesced near the tip of the tree (Fig. 2), also indicating recent origin of the coalescence events. Geological evidence has shown that during the early Pleistocene, a 20,000 year warm period followed ice ages which occurred at regular intervals of ca 100, 000 years (Milankovich cycles; Bennettt, 1990). As with other ferns that produce abundant, very small, wind-dispersed spores (van Zanten, 1978), *A. podophylla* may be expected to periodically expand its populations accompanying climate oscillations. Further estimating the time of expansion of *A. podophylla* populations in Hainan and Guangdong based on calibrated rate of nucleotide substitution and pairwise population divergence of cpDNA *atp*B-*rbc*L sequences will be helpful for deducing the historic demography of the species.

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